

**BONE MORPHOGENETIC PROTEIN-2 IN THE TREATMENT AND
DIAGNOSIS OF CANCER**

[0001] This application claims the benefit of U.S. Provisional Application No.

5 60/261,252 (Langenfeld), filed January 12, 2001.

FIELD OF USE

[0002] The present invention relates to the fields of molecular biology,
immunology, and medicine and provides methods for the treatment and diagnosis of
10 cancer. Specifically, it relates to the use of bone morphogenetic protein-2 (BMP-2),
which is overexpressed in many common cancers as 1) a target for cancer treatment
therapies and 2) a means to diagnose cancer.

BACKGROUND OF THE INVENTION

15 [0003] Various publications or patents are referred to in parentheses throughout
this application. Each of these publications or patents is incorporated by reference
herein. Complete citations of scientific publications are set forth in the text or at the end
of the specification.

[0004] Lung cancer is the leading cause of cancer deaths in the United States with
20 over 150,000 people this year expected to die from this disease (1). Despite
improvements in diagnosis and treatment, only 10% of lung cancer patients survive 5
years (1) with the majority of patients succumbing due to spread of the tumor to other
parts of the body. The genes that induce the invasion and metastasis of lung cancers are

poorly understood. Applicant's experiments to identify genes that regulate metastasis revealed that bone morphogenetic protein-2 (BMP-2) is overexpressed in human lung carcinomas. Subsequent experiments revealed that the protein is also overexpressed in many other common human cancers.

5 [0005] BMP-2 is a powerful morphogenetic protein that has been studied predominantly for its role in embryonic development and its ability to induce bone formation. The bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF) superfamily, which are a phylogenetically conserved group of proteins (2). There are 20 isotypes of the BMPs with BMP-2 and BMP-4 sharing 92%
10 homology (3). BMP-2 and BMP-4 are secreted proteins that induce pluripotential mesenchymal differentiation (4, 5) (6) and are required for the normal embryonic development of many organs including lung and bone (7, 8). BMP-2 can induce the entire developmental program of endochondral osteogenesis when introduced at an ectopic site (9). BMP-2 and BMP-4 also have chemotactic properties capable of inducing
15 the migration of normal vascular endothelial and mononuclear cells (10, 11).

[0006] The BMPs are synthesized as inactive variable length precursor proteins (12, 13). The precursor BMP-2 proteins are proteolytically cleaved, producing a mature C-terminal 14-kDa protein that is the active peptide (9, 12). The mature BMP-2 protein signaling is mediated by transmembrane serine/threonine kinases called type IA, IB, and
20 type II receptors (14-17). The receptor phosphorylates cytoplasmic targets, which includes the Smad family of proteins (18).

[0007] While BMP-2 expression has been noted in a few cancers, such as sarcomas (Guo, W., et al. "Expression of bone morphogenetic proteins and receptors in

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sarcomas" Clin. Orthop. 365: 175-83 (1999)) and pancreatic cancer (Kleef, J., "Bone Morphogenetic Protein-2 exerts diverse effects on cell growth in vitro and is expressed in human pancreatic cancer in vivo" Gastroenterology 116: 1202-1216 (1999)) and in cancer cell lines (Hatakeyama, S., et al., "Expression of bone morphogenetic proteins of human neoplastic epithelial cells" Biochem Mol. Biol. Int. 42(3): 497 (1997)), inhibition of BMP-2 activity as a potential cancer treatment has neither been mentioned nor studied in the literature. To the contrary, several articles suggest that BMP-2 has an inhibitory effect on cancer cell proliferation and may be a useful therapeutic agent to treat cancer. (Kawamura, C., et al., "Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3" Blood 96(6): 2005-11 (2000); Soda, H. "Antiproliferative effects of recombinant human bone morphogenetic protein-2 on human tumor colony-forming units" Anticancer Drugs 9(4): 327-31 (1998); Tada, A., et al., "Bone morphogenetic protein-2 suppresses the transformed phenotype and restores actin microfilaments of human lung carcinoma A549 cells" Oncol. Rep. 5(5): 137-40 (1998))

15 **[0008]** Applicant has discovered that expression of bone morphogenetic protein-2 (BMP-2) is linked to cancer invasion and growth and that inhibiting BMP-2 activity reduces the size of cancerous tumors in nude mice and down regulates the expression of VEGF and sonic hedgehog in lung cancer cell lines. Thus, the present invention is directed toward using BMP-2 as a target for cancer treatment therapies and as a means to

20 diagnose cancer. Specifically, the therapeutic component of this invention involves administering to a patient a composition that inhibits bone morphogenetic protein-2 activity. The diagnostic component of the invention involves measuring the BMP-2 level in biological samples from both a patient and a non-cancerous subject and comparing

those levels, with elevated levels indicating cancer in the patient.

SUMMARY OF THE INVENTION

[0009] The present invention is related to Applicant's discovery that bone morphogenetic protein-2 (BMP-2) is overexpressed in many common human cancers and is linked to cancer invasion and growth. Further, inhibiting BMP-2 activity reduces the size of cancerous tumors in nude mice and down regulates the expression of VEGF and sonic hedgehog in lung cancer cell lines. Thus, the present invention pertains to the use of BMP-2 as a 1) a target for cancer treatment therapies and 2) a means to diagnose cancer.

10 [0010] A primary aspect of the present invention is to provide a method for the treatment of cancer by administering to a patient a therapeutically effective amount of a BMP-2 activity inhibitor. Some cancers that may be treated by this method are carcinomas, including, but not limited to, lung cancer, bladder cancer, breast cancer, colon cancer, kidney cancer, lung cancer, ovarian cancer, thyroid cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer. In a preferred embodiment of this invention the patient is human.

[0011] The BMP-2 inhibitor of this invention may be a polypeptide that binds specifically to bone morphogenetic protein-2, a polypeptide that binds specifically to a BMP-2 receptor, or an antibody that binds specifically to BMP-2. The BMP-2 inhibitor may also be an antisense oligonucleotide that binds to a BMP-2 nucleic acid sequence or some portion thereof.

[0012] This invention features several particular polypeptides that are BMP-2 inhibitors. Preferred embodiments of this invention feature known antagonists to BMP-2,

such as noggin, chordin, cerberus 1 homolog, gremlin, and DAN. Noggin is particularly preferred. Another aspect of this invention relates to the use of fragments of noggin, chordin, cerberus 1 homolog, gremlin, and DAN as BMP-2 inhibitors.

[0013] Another embodiment of this invention provides a method for treating
5 cancer by administering to a patient a therapeutically effective amount of an expression vector encoding a BMP-2 inhibitor, such as a polypeptide that binds BMP-2 or an antisense oligonucleotide that binds to the nucleic acid for BMP-2. Another aspect of this invention includes the expression vector described above in which the nucleic acid sequence for BMP-2 is operably linked to a selective promoter. One preferred selective
10 promoter encompassed by this invention is carcinoembryonic antigen promoter.

[0014] This invention also encompasses a kit that includes packaging material, a BMP-2 activity inhibitor, and instructions that indicate that the compound can be used for treating cancer in a patient. One type of cancer that may be treated is carcinoma. Particular carcinomas encompassed by this invention are lung cancer, bladder cancer,
15 breast cancer, colon cancer, kidney cancer, ovarian cancer, thyroid cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer.

[0015] The diagnostic component of this invention includes a method for diagnosing cancer in a patient by obtaining a biological sample from a patient and measuring the level of BMP-2 in the biological sample, with an elevated level of BMP-2
20 indicating cancer in the patient.

[0016] Any assay available to measure BMP-2 levels is encompassed by this invention. Particularly preferred are immunoassays. Some examples of immunoassays included in this invention are Enzyme-Linked Immunosorbent Assay (ELISA), Western

blot, immunoprecipitation, in situ immunohistochemistry, and immunofluorescence. The Enzyme-Linked Immunosorbent Assay is most particularly preferred.

[0017] Another aspect of this invention is a method for the diagnosis of cancer in a patient by detecting overexpression of BMP-2 in the patient by (i) quantifying *in vivo* or
5 *in vitro* the presence of BMP-2 in a patient or a biological sample obtained from a patient, (ii) comparing the result obtained in step (i) to that of a normal, non-cancerous patient, and (iii) diagnosing for the presence of cancer based on an increased level of BMP-2 in step (ii) relative to a normal, non-cancerous patient.

BRIEF DESCRIPTION OF THE FIGURES

10 [0018] Figure 1 illustrates representational difference analysis (RDA) subtraction. Figure 1(a) shows amplification of cDNA prior to subtraction. Lane 1: IHBE cells; lane 2: lung carcinoma. Figure 1(b) shows the distinct cDNA bands present after the second round of subtraction and amplification. Figure 1(c) lists the proteins that were identified by a BLAST data base search after the DNA corresponding to each of the bands shown in
15 Figure 1(b) was isolated and sequenced.

[0019] Figure 2 is an ethidium-stained agarose gel showing the results of RT-PCR performed on human lung cancer specimens. Lanes 1-4 contain the results of the RT-PCR of various specimens, while lane 5 contains a marker.

[0020] Figure 3 illustrates Western blots showing mature BMP-2 overexpressed
20 in lung cancer tissue specimens and lung cancer cell lines. Figure 3(a) is a representative Western blot showing overexpression of BMP-2 in cancer tissue specimens. Lanes 1-5: normal lung tissue, lane 6: SOAS osteosarcoma cell line, lanes 7-11: non-small lung cell carcinomas. Figure 3(b) is the corresponding actin immunoblot. Figure 3(c) is a Western

blot of non small cell lung carcinoma (NSCLC) subtypes. Lanes 1-4: normal lung tissue, lane 5: squamous carcinoma, lane 6: adenocarcinoma, lane 7: bronchoalveolar carcinoma, lane 8: large cell carcinoma. Figure 3(d) is the corresponding actin immunoblot. Figure 3(e) is a BMP-2 immunoblot of lane 1: benign lung tumor, lane 2: mesthotheleoma, lane 3: normal lung tissue, lane 4: carcinoid tumor, lane 5: normal lung, lane 6: NSCLC, lane 7: normal lung tissue, lane 8: NSCLC, lane 9: recombinant BMP-4. Figure 3(f) is a BMP-4-probed Western blot with the same lane contents as Figure 3(e), except lane 9, which is recombinant BMP-4. Figure 3(g) is the corresponding actin immunoblot.

10 [0021] Figure 4(a) is a Western immunoblot of total cellular protein that demonstrates that normal and malignant human lung cell lines express mature BMP-2 protein. Lanes (1) IHBE; (2) SOAS; (3) H7249; (4) A549. (b) Western blot of cell culture media shows lung cancer cell lines secrete a BMP-2 precursor protein. Lanes (1) lung cancer tumor specimen; (2) A549 media; (3) H7249 media; (4) IHBE; (5), NBE media; 15 (6) serum free media alone. (c) immunoblot of BMP type IA receptor. Lanes (1-3) normal lung tissue specimens; (4) IHBE cells; (5) H7249 cells; (6) A549 cells; (7-9) lung cancer tissue specimens.(d) immunoblot of BMP type IB receptor. (1-3) normal lung tissue specimens; (4) IHBE cells; (5)H7249 cells; (6) A549 cells; (7-9) lung cancer tissue specimens.

20 [0022] Figure 5: 5(a) is an immunohistochemistry localizing BMP-2 expression to the tumor cells. BMP-2 expression in a NSCLC demonstrating cytoplasmic staining of the tumor cells (arrowheads). The nuclei (n) of the tumor cells and the interstitium (I) are non-reactive; (b) Preabsorption of the BMP-2 antibody with recombinant human BMP-2

is non-reactive with the tumor cells (arrows). Original magnification x 82 .

[0023] Figure 6(a) is a BMP-2 Western blot of human breast tumors and corresponding normal tissue. Lane 1: NSCLC, lane 2-5: breast carcinomas, lane 6-8: normal breast tissue, lane 9: recombinant BMP-2. Figure 6(b) is a BMP-2 Western blot of common human carcinomas and the corresponding normal tissue. Lane 1: normal endometrium, lane 2: endometrial carcinoma, lane 3: ovarian carcinoma, lane 4: normal colon, lane 5: colon carcinoma, lane 6: normal bladder, lane 7: bladder carcinoma.

[0024] Figure 7(a) is a Western blot showing BMP-2 expression in metastatic tumors. Lane 1: interstitial inflammatory lung disease, lane 2: normal omentum, lane 3: metastatic kidney tumor, lane 4: normal lymph node, lane 5: metastatic breast cancer, lane 6: metastatic kidney tumor, lane 7: metastatic NSCLC, lane 8: omentum carcinoma. Figure 7(b) is the corresponding actin immunoblot. Figure 7(c) is a BMPR IA Western blot, while Figure 7(d) is a BMPR IB Western blot. The contents of the lanes on both blots are the same: lane 1: normal kidney, lanes 2-3: normal lung, lane 4: metastatic kidney carcinoma, lane 5: metastatic breast carcinoma, lane 6: metastatic NSCLC, lanes 7-9: NSCLC. Figure 7(e) is BMPR IA Western blot and Figure 7(f) is a BMPR IB Western blot of common human carcinomas. Lane contents are the same on both blots: lane 1: normal kidney, lane 2: normal endometrium, lane 3: omentum, lane 4: normal colon, lane 5: ovarian carcinoma, lane 6: kidney carcinoma, lane 7: endometrial carcinoma, lane 8: omental tumor, lane 9: colon carcinoma.

[0025] Figure 8 is a Western blot showing BMP-2 in serum samples from lung cancer patients. Lanes 1-2: serum samples, lane 3: recombinant BMP-2.

[0026] Figure 9 shows that secreted BMP-2 precursor is proteolytically cleaved

by human leukocytes. Cell culture media from the A549 cells incubated with leukocytes for 16 hours is probed with BMP-2 antibody recognizing its mature C-terminal end.

Figure 9(a) is the resulting Western blot: lane 1: A549 lysate, lane 2: media without leukocytes, lanes 3-4: media with human leukocytes. Figure 9(b) is the same

5 immunoblot hybridized with BMP-2 antibody recognizing its N-terminal end. Figure 9(c) is a Western blot of leukocyte samples probed with anti-furin antibody.

[0027] Figure 10 shows that BMP-2 treatment enhances formation of blood vessels around a cancerous tumor. Each picture is of tissue from a nude mouse injected either with A549 cells or with A549 cells and BMP-2. The picture in the upper right
10 shows tissue (including a tumor) from a nude mouse injected with A549 cells. Upper left: control. Upper right: mouse treated with BMP-2. Lower left: mouse treated with noggin.

[0028] Figure 11 shows tissue (from nude mice injected with A549 cells and nude mice co-injected with A549 cells and BMP-2) stained with anti-CD 31 antibody, which
15 recognizes endothelial cells, viewed from under a microscope. Left: control. Right: BMP-2 treated.

[0029] Figure 12 shows that BMP-2 regulates sonic hedgehog expression. The Western blot on the left was probed with anti sonic hedgehog and shows an increase in sonic hedgehog expression as the amount of recombinant BMP-2 added to the A549 cell
20 culture is increased. The Western blot on the right was probed with anti sonic hedgehog and shows A549 cell culture media without added noggin (Lane 1) and cell culture media with added noggin (Lane 2).

[0030] Figure 13 shows that BMP-2 stimulates the migration of A549 and H7249

human lung cancer cell lines. 13(a): Recombinant human BMP-2, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml was added to the lower well of the transwell chamber.

Migrated cells counted using fluorescent microscopy. 13(b) Noggin inhibits BMP-2 induced migration. Lane (1), media alone; (2) recombinant BMP2 (500 ng/ml); (3)

5 noggin (10 mg/ml) and recombinant BMP-2 (500 ng/ml). 13(c) H7249 cells migrated off cover slips towards Affi-Blue agarose beads containing recombinant BMP-2. 13(d)

H7249 cells did not migrate off cover slips toward AffiBlue agarose beads containing dilution buffer. Similar results were found using the A549 cells. All the above experiments were repeated at least 3 times. Data presented as mean + standard deviation.

10 13(e): Recombinant human BMP-2 stimulates the invasion of A549 or H7249 cells.

Recombinant BMP-2, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml was added to the lower wells of a Matrigel invasion chamber. Experiments were repeated at least 3 times. Data presented as mean + 5 standard deviation.

[0031] Figure 14(a) show tumor growth after 19 days following the subcutaneous
15 co-injection of A549 lung cancer cells into nude mice with Affi-blue agarose beads coated with (1) 100 ug/ml of albumin, (2) recombinant human BMP-2, or (3) recombinant mouse noggin.

[0032] Figure 15 show that noggin inhibits VEGF expression in the A549 lung cancer cell line. The Western blot was probed with anti-VEGF antibody. The lane
20 labeled with a plus was cell culture media from cultures treated with noggin. The lane labeled with a minus was cell culture media from control cultures.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The present invention is related to Applicant's discovery that the

overexpression of bone morphogenetic protein-2 (BMP-2) is linked to cancer invasion and growth. BMP-2 is overexpressed in many common human cancers and regulates molecular pathways that are involved in the promotion of cancer. Inhibiting BMP-2 activity reduces the size of cancerous tumors in nude mice and down regulates the expression of VEGF and sonic hedgehog, which have been linked to cancer, in lung cancer cell lines. Thus, the present invention is directed toward using BMP-2 as a target for cancer treatment therapies and as a means to diagnose cancer.

[0034] The therapeutic component of this invention involves administering to a patient a composition that inhibits bone morphogenetic protein-2 activity. Such inhibition may be accomplished by ligands or antibodies that bind to BMP-2 or BMP-2 receptors. It may also be achieved by preventing the processing of pro-BMP-2, or blocking transcription or replication of BMP-2 DNA or translation of BMP-2 mRNA. Delivery of such compositions may be systemic or tissue-targeted.

[0035] The diagnostic component of the invention involves measuring the BMP-2 level in biological samples from both a patient and a non-cancerous subject and comparing those levels. Elevated levels of BMP-2 in the patient compared to the subject indicate cancer.

[0036] Although specific embodiments of the present invention will now be described, it should be understood that such embodiments are examples that are merely illustrative of a small number of the many possible specific embodiments that can represent applications of the principles of the present invention. Various modifications obvious to one skilled in the art to which the present invention pertains are within the spirit, scope and contemplation of the present invention as further defined in the

appended claims.

Definitions

[0037] A “bone morphogenetic protein-2 activity inhibitor” is a composition that antagonizes the activity of the BMP-2 protein by specifically binding to it or to BMP
5 receptors, blocks the activation of pro-BMP-2, or prevents the replication or transcription of the BMP-2 gene or the translation of BMP-2 mRNA into protein.

[0038] “Polypeptide” refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds.
Polypeptide refers to both short chains, commonly referred to as peptides, oligopeptides
10 or oligomers, and to longer chains, generally referred to as proteins. Polypeptides include amino acid sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature.

15 [0039] An “expression vector” is a recombinant vector that incorporates the desired gene and associated control sequences that promote and/or regulate expression of the gene. The desired gene is “operably linked” to such control sequences. The term “operably linked” means that the regulatory sequences necessary for expression of the coding sequence are placed in an appropriate position in the expression vector relative to
20 the coding sequence so as to enable expression of the coding sequence. The preparation of such recombinant expression vectors as well as the use of various control sequences is well known to those of skill in the art and described in many references. See, for example, Sambrook, J., et al., Molecular Cloning : A Laboratory Manual 2nd ed. (Cold

Spring Harbor, N.Y., Cold Spring Harbor Laboratory) (1989).

[0040] A “selective promoter” refers to a promoter that is not indiscriminately expressed. Instead it is expressed only, for example, in certain tissues, certain tumors, in response to certain treatments, or in response to certain events in a cell. Such tissue-specific, tumor-selective, treatment-responsive, or tumor endothelium directed promoters are described in Nettlebeck, D.M., et al., “Gene therapy: designer promoters for tumour targeting” Trends Genet 16(4); 174-81 (2000).

[0041] An “expression vector vehicle” refers to an expression vector paired with a moiety that facilitates delivery of the expression construct to cells *in vivo*. An expression vector may incorporate genes encoding the delivery moiety. One example of such an expression vector is a viral vector.

[0042] The term “antibody” refers to polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of a Fab or other immunoglobulin expression library.

[0043] “Polyclonal” refers to antibodies that are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen or antigenic functional derivative thereof. For the production of polyclonal antibodies, various host animals may be immunized by injection with the antigen. Various adjuvants may be used to increase the immunological response, especially when using an entire protein, or a larger section of the protein. The type of adjuvant used will depend on the hosts. Typical adjuvants include Freund’s, Freund’s complete, or oil-in-water emulsions. In these cases the entire protein or portion thereof can serve as the antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger

molecule to make an immunostimulatory conjugate for use as the antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH).

[0044] "Monoclonal antibodies" are substantially homogeneous populations of antibodies to a particular antigen. They may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. Such methods are well known to those of ordinary skill in the art and include general hybridoma methods of Kohler and Milstein, Nature (1975) 256: 495-497, the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72 (1983) and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, pp.77-96, Alan R. Liss, Inc. (1985). The basic technique involves injecting a mouse, or other suitable animal, with an antigen. The animal is subsequently sacrificed and cells taken from its spleen are fused with myeloma cells. The result is a hybrid cell, referred to as a hybridoma, that reproduces in vitro. The population of hybridomas are screened to isolate individual clones each of which secrete a single antibody species to the antigen. The individual antibody species obtained in this way are each the product of a single B cell from the immune animal generated in response to a specific antigenic site recognized on the antigen. Kohler, G. and Milstein, C. Nature (London) 256: 495-497 (1975) and Eur. J. Immunol. 6: 511-519 (1976).

[0045] The term "antibody fragment" refers to a portion of an antibody, often the hyper variable region and portions of the surrounding heavy and light chains, that displays specific binding affinity for a particular molecule. The term antibody fragment also includes single chain antibodies.

[0046] An “antisense oligonucleotide” is an oligonucleotide that specifically hybridizes, under cellular conditions, with the cellular mRNA or genomic DNA encoding a BMP-2 protein or some portion of such cellular or genomic DNA, thereby inhibiting biosynthesis of the BMP-2 protein. The binding may be via conventional base pair
5 complementarity, or, in the case of binding to DNA duplexes, via specific interactions in the major groove of the double helix.

[0047] The term “effective amount” refers to the quantity of a compound that is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk
10 ratio when used in the manner of this invention. The specific “effective amount” will, obviously, vary with such factors as the particular cancer being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or their derivatives.

15 [0048] A “patient” is a mammal suspected of having cancer. The patient is preferably human but may also be another mammal, such as a cat, dog, horse, cow, rat, or mouse.

[0049] A “biological sample” is a substance obtained from the patient’s body. The particular “biological sample” selected will vary based on the cancer the patient is
20 suspected of having and, accordingly, which biological sample is most likely to contain BMP-2.

[0050] An “elevated level” means the level of bone morphogenetic protein-2 that is greater than the level of analyte present in a particular biological sample of patient that

is not suffering from cancer.

[0051] A "carcinoma" is an epithelial cancer. Examples of carcinomas are bladder cancer, breast cancer, colon cancer, kidney cancer, lung cancer, ovarian cancer, thyroid cancer, endometrial cancer, omental cancer, testicular cancer, and liver cancer.

- 5 The epithelium predominately lines ducts and lining of organs or glands.

BMP-2 as a Target in the Treatment of Cancer

[0052] The present invention is directed to the use of BMP-2 as a target in the treatment of cancer. Amino acids #283-396 of SEQ ID NO: 2 constitute the amino acid sequence of mature human BMP-2. Nucleotides #372-1514 of SEQ ID NO: 1 constitute
10 the nucleotide coding sequence for human BMP-2. Given the experiments described above, any composition that 1) specifically binds BMP-2 or a BMP-2 receptor, thereby antagonizing BMP-2 activity, 2) blocks the processing of pro-BMP-2, or 3) prevents the replication or transcription of BMP-2 DNA or the translation of BMP-2 mRNA could be used as a therapy to treat cancer.

- 15 [0053] A compound that specifically binds to BMP-2 is any compound (such as a polypeptide or an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of BMP-2. As one of ordinary skill in the art will appreciate, such "specific" binding compounds may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more
20 preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of BMP-2.

[0054] Similarly, a compound that specifically binds to a BMP receptor is any compound that has a binding affinity for any naturally occurring isoform, splice variant,

or polymorphism of the BMP receptor. As one of ordinary skill in the art will appreciate, such “specific” binding compounds may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid
5 sequence of a BMP receptor.

[0055] The present invention embodies polypeptides that specifically bind to BMP-2, thereby inhibiting its activity or that specifically bind to BMP receptors, thereby inhibiting BMP-2 activity. Specific embodiments of such polypeptides are described below.

10 [0056] The present invention encompasses known antagonists of BMP-2 activity, including noggin (Brunet, L.J., et al., “Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton” Science 280(5368): 1455-7 (1998); US Patent No. 6,075,007, Economides, et al.), chordin (US Patent No. 5,896,056, LaVallie, et al.; Millet, C., et al., “The human chordin gene encodes several differentially spliced variants
15 with distinct BMP opposing activities” *Mech. Dev.* 106(1-2): 85-96 (2001)), gremlin (GenBank Accession No. AF154054), cerberus 1 homolog (GenBank Accession No. NM_005454), and DAN.

[0057] Recombinant mouse noggin from R & D Systems (Minneapolis, MN) was used in the inhibition experiments described in the Results section below. Mouse and
20 human noggin share 98% homology. Therefore, this invention also relates to use of a polypeptide with the amino acid sequence of mature mouse noggin (amino acids #20-231 of SEQ ID NO: 6) and with the amino acid sequence of mature human noggin (amino acids #20-231 of SEQ ID No.: 4) as a BMP-2 activity inhibitor. The amino acid

sequence for human chordin is SEQ ID No: 8, for human gremlin is SEQ ID NO: 10, and
for cerberus 1 homolog is SEQ ID NO: 12. The nucleotide coding sequence for human
noggin is SEQ ID NO: 3, for mouse noggin is SEQ ID NO: 5, for human chordin is
nucleotides #247-3114 of SEQ ID NO: 7, for human gremlin is nucleotides #130-684 of
5 SEQ ID NO: 9, for human cerberus 1 homolog is SEQ ID NO: 11.

[0058] This invention also embodies polypeptide fragments of noggin, chordin,
gremlin, cerberus1 homolog, and DAN that bind BMP-2 and inhibit its activity. Such
polypeptides may be tested for inhibitory efficiency by culturing cells transformed with
progressively shorter portions of the nucleotide sequences encoding the above proteins,
10 recovering and purifying from the various cultures the resulting polypeptide, and testing
those polypeptides for their ability to inhibit BMP-2 activity.

[0059] This invention also includes genetically altered BMP receptor proteins that
inhibit BMP-2 activity. For example, altered BMP receptors that inhibit the binding
effects of BMP-2 are described in U.S. Patent No. 6,291,206 (Wozney, et al.)

15 [0060] Also included by this invention are polypeptides that bind BMP receptors
without activating them. (Nickel, J., et al. "The Crystal Structure of the BMP-2:BMPR-
IA Complex and the Generation of BMP-2 Antagonists" The Journal of Bone & Joint
Surgery 83-A, Supp.1, Part 1: 7-14 (2001). Kirsch, T., et al. "BMP-2 antagonists emerge
from alterations in the low-affinity binding epitope for receptor BMPR-II" The EMBO
20 Journal 19(13):3314-24 (2000)) Particularly preferred are ligands that will bind BMP IB
receptors, as aberrant expression of the BMP IB receptor in many human cancer
specimens has been noted, as discussed in the Results section below. (Ide, H., et al.,
"Cloning of human bone morphogenetic protein type IB receptor (BMPR-IB) and its

expression in prostate cancer in comparison with other BMPRs" Oncogene 13(11): 1377-82 (1997)). The coding sequence for BMP IB precursor is nucleotides #274-1782 of SEQ ID NO: 13. The amino acid sequence for BMP IB is amino acids #14-502 of SEQ ID NO 14.

- 5 **[0061]** This invention also encompasses expression vectors that incorporate a nucleotide sequence encoding an inhibitor of BMP-2 activity operably linked to control sequences that promote and/or regulate expression of the nucleotide sequence. The preparation of such expression vectors as well as the use of various control sequences is well known to those of skill in the art and is described in many references, such as
- 10 Sambrook, et al. (1989). Expression vectors can be derived from bacterial plasmids, from bacteriophage, from transposon, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses and from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Promoters can be prokaryotic, such as lacI, lacZ, T3, T7, gpt, lambda PR,
- 15 PL, and trp, or eukaryotic, such as CMV immediate early, HSV thymidine kinase, early and late SV40, LTR's from retrovirus, and mouse metallothionein-1. Selective promoters such as those described in Nettlebeck, D.M., et al., "Gene therapy: designer promoters for tumour targeting" Trends Genet 16(4); 174-81 (2000) that are tissue-specific, tumor-selective, treatment-responsive, or tumor endothelium directed may also be used. For
- 20 example, the promoter of the carcinoembryonic antigen (CEA) is expressed on many breast, lung, and colorectal cancers.

[0062] For introduction of a gene that encodes a protein that antagonizes BMP-2 activity an expression vector vehicle that will facilitate delivery of the desired gene to the

affected cells may be used. One way to facilitate delivery is by using an expression vector derived from virus. Examples of viral vectors that have been successfully used to deliver desired sequences to cells with high infection efficiency are adenoviral, retroviral, vaccinia viral, and adeno-associated viral vectors. Commonly used viral promoters for expression vectors are derived from polyoma, cytomegalovirus, Adenovirus, and Simian Virus 40 (SV40). It is also possible to use promoter or control sequences normally associated with the desired gene sequence, if such control sequences are compatible with the host cell systems.

[0063] Non-viral expression vector vehicles are also available. For instance, the expression vector could be associated with one or more lipids. As is known in the art of lipid-based gene delivery, such nucleic acid –lipid complexes can be in a variety of different forms depending generally on the nature of the lipid employed, the ratio of nucleic acid to lipid and /or other possible components, and the method by which the complex is formed. Examples of complexes include liposomes and micelles. Liposome-mediated gene transfer seems to have great potential for certain in vivo applications in animals. Studies have shown that intravenously injected liposomes are taken up essentially in the liver and the spleen, by the macrophages of the reticuloendothelial system. Using a catheter to introduce liposomes coupled to expression vectors to particular cellular sites has also been described. (Nabel, E.G., et al., Science 249:1285-1288 (1990))

[0064] Another possible expression vector vehicle consists of a cell receptor-specific ligand and a DNA-binding agent that would bind to the expression vector. (Nishikawa, M. et al., Gene Therapy 7:548-55 (2000)). Such a vehicle could also

comprise a cell receptor-specific ligand and the nucleic acid-lipid complex described above. (Nicolau, C. et al., Methods Enzymol 149: 157-76 (1987))

[0065] In addition, the present invention embodies antibodies that specifically bind BMP-2 or BMP receptors, thereby inhibiting BMP-2 activity. When raising
5 antibodies to BMP-2 or BMP receptors, the entire protein (either the precursor or the processed protein), or a portion thereof, may be utilized. Information useful in designing an antigen for the production of antibodies to BMP-2 may be deduced by those of skill in the art by homology analysis of SEQ ID NO: 2, especially amino acids #283-396 of SEQ ID NO: 2.

10 [0066] A recombinant human BMP-2 protein is commercially available from R & D Systems (Minneapolis, MN) and portions of the BMP-2 protein may be produced by a variety of methods. In order to raise antibodies to particular epitopes, peptides derived from the full BMP-2 sequence may be used. Custom-synthesized peptides in the range of 10-20 amino acids are available from a multitude of vendors, and can be ordered
15 conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a recombinant protein production system. In order to ensure proper protein glycosylation and processing an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred.

20 [0067] Selection of antibodies which alter the activity of BMP-2 may be accomplished in several ways. Antibodies that alter the binding of BMP-2 to a receptor may be detected by well known binding inhibition assays. For instance, according to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled)

antibody to BMP-2, which has been immobilized in a microtiter well, is assayed for BMP-2 binding in both the presence and absence of the appropriate receptor. The decrease in binding will be indicative of a competitive inhibitor relationship between the antibody and the receptor. In addition, antibodies that are useful for altering the function of BMP-2 may be assayed in functional formats, such as the cell migration assays described in the Results and Examples sections.

[0068] This invention also embodies compositions that prevent the processing of inactive BMP-2 precursors. BMP precursors are proteolytically activated by proprotein convertases. For example, BMP-2 is cleaved by furin convertase from human leukocytes. Furin inhibitors are known. See, e.g., Cameron, A., et al., "Polyarginines are potent furin inhibitors" J. Biol. Chem. 275: 36741-49 (2000).

[0069] While the BMP-2 inhibitors discussed above adversely affect BMP-2 activity after it is expressed, it will be readily apparent to one of ordinary skill in the art that specific prevention of BMP-2 biosynthesis will achieve the same goals as more direct inhibition of its activity. Consequently, this invention also encompasses inhibition of BMP-2 biosynthesis as a method for treating cancer. Such inhibition may be achieved by selectively degrading mRNA encoding BMP-2 or by interfering with transcription or translation of such mRNA. See Glavic, A., et al., "Xiro-1 controls mesoderm patterning by repressing BMP-4 expression in the Spemann organizer" Dev. Dyn. 222(3): 368-376. As mentioned above, BMP-2 shares 92% homology with BMP-4.

[0070] Inhibition of BMP-2 biosynthesis to treat for cancer could also be achieved through antisense therapy. Antisense therapy is the administration or in situ generation of oligonucleotides that specifically hybridizes, under cellular conditions, with

the cellular mRNA or genomic DNA encoding a BMP-2 protein or some portion of such cellular or genomic DNA, thereby inhibiting biosynthesis of the BMP-2 protein.

Antisense therapy refers generally to the range of techniques known by one of ordinary skill in the art, and includes any therapy that relies on specific binding to oligonucleotide
5 sequences.

[0071] Delivery of an antisense oligonucleotide of the present invention can occur in a variety of ways. For example, an antisense oligonucleotide can be delivered as an expression vector that produces RNA which is complementary to at least a unique portion of the cellular mRNA encoding BMP-2. Such an expression vector could be delivered to
10 cells by one of the expression vector vehicles described above. Alternatively, the antisense oligonucleotide could be generated ex vivo as an oligonucleotide probe which, when introduced to the cell, inhibits biosynthesis of BMP-2 proteins by hybridizing with the mRNA or genomic sequences encoding BMP-2. Such oligonucleotide probes could be modified oligonucleotides that are resistant to endogenous nucleases and therefore are
15 stable in vivo. General methods to construct oligomers useful in antisense therapy are known in the art. (Van der krol, et al., Biotechniques 6:958-976 (1988); Stein, et al., Cancer Res. 48:2659-2668 (1988).

[0072] Dosage forms of the BMP-2 inhibitors of this invention include pharmaceutically acceptable carriers known to those of ordinary skill in the art.

20 Pharmaceutically acceptable components are those that are suitable for use with mammals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio. The carrier can be a solid or liquid and the type is generally chosen based on the type of administration being used.

The active agent can be coadministered in the form of a tablet or capsule, as an agglomerated powder or in a liquid form. Examples of suitable solid carriers include lactose, sucrose, gelatin and agar. Capsule or tablets can be easily formulated and can be made easy to swallow or chew; other solid forms include granules and bulk powders.

- 5 Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents and melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/ or suspension reconstituted from non-
- 10 effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents, sweeteners, thickeners and melting agents. Parenteral and intravenous forms may also include isotonic salts and other materials to make them compatible with the type of injection or delivery system chosen.

- 15 **[0073]** For administration of an antibody to BMP-2, the pharmaceutically acceptable carrier will usually be an aqueous solution, such as normal saline or phosphate-buffered saline (PBS), Ringer's solution, lactate-Ringer's solution, or any isotonic physiologically acceptable solution for administration by the chosen means. In addition to additives for adjusting pH or tonicity, the antibody may be stabilized against
- 20 aggregation and polymerization with amino acids and non-ionic detergents, polysorbate, and polyethylene glycol. Optionally, additional stabilizers may include various physiologically-acceptable carbohydrates and salts. Also, polyvinylpyrrolidone may be added in addition to the amino acid. Suitable therapeutic immunoglobulin solutions,

which are stabilized for storage and administration to humans are described in U.S.

Patent No. 5,945,098. Other agents, such as human serum albumin (HAS), may be added to the pharmaceutical composition to stabilize the antibody conjugates.

[0074] The method of administration can be any suitable method that effectively
5 alleviates the particular cancer being treated. Possible methods of administration are oral, rectal, parenteral, enterical, subcutaneous, transdermal, peritoneal, intratumoral, or intravenous.

[0075] Any suitable dosage of the compounds may be given in the method of the invention. Dosage levels and requirements are well-recognized by those of ordinary skill
10 in the art. As one of ordinary skill in the art will appreciate, an amount constituting an effective amount will vary depending on particular factors. For instance, specific dosage and treatment regimens will depend on facts such as the patient's general health profile, the type of cancer being treated, the severity and course of the patient's disorder, other therapeutics being administered to treat the cancer, and the judgment of the treating
15 physician.

[0076] The present invention also provides kits for treating cancer using BMP-2 activity inhibitors. For example, such kits can comprise any one or more of the following materials: packaging material, at least one type of BMP-2 activity inhibitor, and instructions regarding dosage, method of administration, or the like for using the inhibitor
20 to treat cancer.

Detection of BMP-2 to Aid in Diagnosis of Cancer

[0077] In addition to its therapeutic aspects, the present invention also relates to a diagnostic method for detecting the presence of elevated levels of BMP-2 in the patient.

Applicants have shown that BMP-2 is expressed in many common cancers. Elevated levels of BMP-2 can be detected in various biological samples in mammals, preferably humans. Applicants have shown the presence of BMP-2 in the blood serum of a human patient with cancer. Biological samples, including but not limited to blood, vitreous humor, sputum, aqueous humor, synovial fluid, urine, ascites, and tissue, will be drawn from the patient using standard techniques. Particularly preferred are serum samples.

[0078] The measurement of BMP-2 levels may be monitored using any method possible to detect BMP-2 in biological samples. Immunoassays, such as Enzyme Linked Immunological Assay (ELISA), Western blots, immunoprecipitation, in situ immunohistochemistry, and immunofluorescence assays are preferred. ELISA is particularly preferred. For a review of general immunoassays, see Stites, D.P., et al., eds., Basic and Clinical Immunology, 8th ed. (Appleton & Lange, Norwalk, Conn.) (1994). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein of choice, BMP-2, in this case. The antibody is generally fixed to a substrate such as a plate or a column via covalent or non-covalent linkages (e.g., streptavidin, protein A, protein G, secondary antibodies). Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may be a labeled anti-BMP-2 antibody. Alternatively, the labeling agent may be a third moiety, such as a secondary antibody, that specifically binds to the antibody/antigen complex.

[0079] The immunoassays of this invention may be competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In a "sandwich" assay, for example, the anti-BMP-2

antibodies can be bound directly to a solid substrate on which they are immobilized.

These immobilized antibodies then capture BMP-2 in the test sample. BMP-2 thus immobilized is then bound by a labeling agent, such as a second antibody bearing a label.

Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a

5 labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, e.g., streptavidin, to provide a detectable moiety. Methods of binding molecules to a solid support, either covalently or non-covalently, are well known to those of skill in the art. A variety of
10 solid supports known to those of skill in the art, e.g., plate, columns, dipsticks, membranes, and the like, can be used with the present invention.

[0080] In competitive assays, the amount of BMP-2 is measured indirectly by measuring the amount of a known modified BMP-2 displaced from a BMP-2 antibody by the unknown BMP-2 in a sample. In one competitive assay, a known amount of modified
15 BMP-2 is added to a sample and the sample is then contacted with an anti-BMP-2 antibody. The amount of known modified BMP-2 bound to the antibody is inversely proportional to the concentration of BMP-2 in the sample. The amount of modified BMP-2 may be detected by providing a labeled modified BMP-2 molecule.

[0081] The label used in the assay is not a critical aspect of the invention, so long
20 as it does not significantly interfere with the specific binding antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Examples of such

labels are magnetic beads, fluorescent dyes, radiolabels, enzymes, and calorimetric labels such as colloidal gold or colored glass or plastic beads.

[0082] The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide
5 variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule, such as biotin, is covalently bound to the molecule. The ligand then binds to another molecule, such as streptavidin, which is either inherently
10 detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize BMP-2. The molecules can also be conjugated directly to a signal generating compound, e.g., by conjugation with an enzyme or fluorophore.

15 [0083] Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be
20 detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers or the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally, simple colorimetric labels may be

detected simply by observing the color associated with the label.

[0084] Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies.

RESULTS

5 [0085] Experimental results supporting the above uses of BMP-2 and its inhibitors are set forth in detail below. All of the experimental methods mentioned in this section, such as representational difference analysis, Western blot assays, and immunohistochemical studies, are described in detail in the Examples section that follows.

10 Identification of BMP-2 Using RDA Subtraction Technique:

[0086] Initially, Applicant performed representational difference analysis (RDA) on cDNA derived from normal and cancerous lung tissue samples to identify genes that were uniquely or highly expressed in human lung cancer in comparison to normal tissue. RDA has been described in the literature and allows detection of differences in gene
15 expression between two similar populations. It involves exposing digested tester cDNA ligated to a primer to high concentrations of similarly digested but non-primer bearing driver cDNA, melting the tester and driver cDNA, and allowing them to hybridize. Subsequent PCR results in exponential amplification of the target cDNA of the tester that hybridizes to other tester cDNA. (Hubank, M., Nucleic Acids Research 22:5640-5648
20 (1994)) Here, Applicant used a non-small cell lung carcinoma (NSCLC) as the tester and immortalized human bronchial epithelial (IHBE) cells as the driver. IHBE cells rather than normal lung tissue were used, as IHBE cells proliferate at a rate that is more similar to human lung carcinomas than to normal lung tissue. Thus, Applicant avoided

identifying genes involved in the proliferation cascade but that were not by themselves transforming.

[0087] After two rounds of subtraction, several distinct bands, which were cloned and sequenced, were present in the amplified tester cDNA. (Figure 1b) A BLAST data base search identified BMP-2 expression in the lung tumor tissue specimen as well as expression of alpha-1-antitrypsin, cytokeratin 6, and lambda light. (Figure 1c)

Expression of BMP-2 In Various Cancer Tissue Specimens, Cancer Cell Lines, and Blood Serum from a Cancer Patient

[0088] Using reverse transcriptase polymerase chain reaction (RT-PCR), Western blots, and immunohistochemical assays to study the expression of BMP-2 and its receptors in various tissue specimens and in cell lines, Applicant found that BMP-2 was highly expressed in many types of cancers.

[0089] Applicant performed his initial experiments on normal and cancerous lung tissue and lung cancer cell lines. RT-PCR was performed using BMP-2 primers and showed expression in 9 out of 10 tumors examined. (Figure 2) Using Western blot analysis, Applicant found that the mature active 14 kD BMP-2 protein was aberrantly expressed in almost all of the 25 non-small cell lung carcinoma (NSCLC) tissue specimens examined. There was little to no expression of BMP-2 in 11 normal lung tissue specimens. A representative Western blot is shown in Figure 3. An anti-actin immunoblot showed near equal loading of the samples. (Figure 3(b)) In addition, BMP-2 was found to be highly expressed in all epithelial derived lung carcinomas of which NSCLC is derived and in the rare malignant neuroendocrine tumor. (Figure 3(c) and Figure 3(e), Lane 4, respectively) Western blot analysis of each of the different cell types

comprising NSCLC – adeno, squamous, large cell, and bronchoalveolar carcinomas –

revealed that the level of BMP-2 expression was not dependent on the cell type or whether the tumor was well or poorly differentiated. In comparison, the level of BMP-2 expression in benign lung tumors (Figure 3(e), Lane 1) and inflammatory diseases of the lung (Figure 7(a), Lane 1) was very low, similar to that seen in normal lung tissue,

5 showing that BMP-2 is not an acute phase protein and that high levels of BMP-2 expression are indicative of malignant tumors. Neither BMP-4 nor BMP-7 expression was detected in the lung tissue specimens or the A549, H7249, IHBE, and NBE cell lines by Western analysis. (Figure 3(f))

[0090] Applicants also tested for expression of BMP-2 in various lung cancer and
10 normal cell lines. Although the mature BMP-2 protein was detected in the cell lysate of the A549 and H7249 human lung cancer cell lines, the level of expression was not significantly different from the level of expression in the cell lysate of immortalized normal human bronchial epithelial cells (IHBE). (Figure 4(a)) Because BMP-2 is a secreted protein, Applicant also examined its expression in the cell culture media. A
15 Western blot of the cell culture media showed the A549 and H7249 cell lines secreted a 43 kD BMP-2 precursor protein. (Figure 4(b), Lanes 2-3) This BMP-2 precursor was not detected in the media from either the IHBE or normal bronchial epithelial (NBE) cells (Figure 4(b), Lanes 4-5).

[0091] Immunohistochemistry studies of patient derived NSCLC also localized
20 the expression of BMP-2 to the cancer cells (Figure 5(a)). Absorbing the anti-BMP-2 antibody with recombinant human BMP-2 completely inhibited staining of the tumors (Figure 5(b)). BMP-2 expression was not detected in normal lung tissue by immunohistochemistry.

[0092] Applicants turned next to receptors and found that normal and cancer lung tissue specimens and cell lines express both type IA and IB BMP receptors. The lung cancer and normal lung tissue specimens express a 55 kD and 44 kD type IA BMP-2 receptor. The tumor specimens expressed predominately the 55 kD receptor, while
5 normal lung tissue specimens expressed a higher percentage of the 44 kD receptor. The A549, H7249, and IHBE cells only expressed a 44 kD type IA BMP receptor. (Figure 4(c)) The tissue specimens and cell lines expressed a 44 kD type IB BMP receptor with normal lung tissue demonstrating more expression than that of the tumor specimens. (Figure 4(d))

10 [0093] Similar to their findings with lung tissue, Applicants found that BMP-2 was expressed in many other common human malignancies but not in their corresponding normal tissues. Western blot analysis revealed that BMP-2 was overexpressed in breast, bladder, colon, endometrial, omental, and kidney carcinomas with low levels of BMP-2 expression in the corresponding normal tissue. (Figures 6(a) and (b)). BMP-2 was also
15 found to be expressed in ovarian (Figure 6(b), lane 3), mesothelioma (Figure 3(e), lane 2), thyroid, hepatoma, and testicular carcinoma.

[0094] BMP-2 and its receptors were also examined in both primary and metastatic carcinomas that were surgically removed from patients. BMP-2 was found to be highly expressed in kidney tumors that had metastasized to the lung, a metastatic
20 breast cancer to chest wall cavity, and a NSCLC lung tumor that had metastasized to a regional lymph node. (Figure 7(a)) The BMP IA receptor was expressed equally between the primary and metastatic carcinomas and the corresponding normal tissue (Figure 7). The BMP IB receptor was expressed in all metastatic and primary tumors

examined. (Figure 7) The BMP IB receptor, in contrast to the BMP IA receptor, was not expressed in all the corresponding normal tissues. While it was expressed in normal lung tissue with slight expression in normal endometrium it was not expressed in normal kidney, colon, and omentum. (Figure 7(f)) Interestingly, the IB receptor was expressed in both primary and metastatic renal carcinoma, but not in normal kidney tissue. (Figure 7(f), Lane 6)

[0095] BMP-2 expression was also found in blood serum samples from lung cancer patients. (Figure 8)

Processing of Inactive BMP-2 Precursors

[0096] Because BMP precursors are proteolytically activated by proprotein convertases, Applicant studied whether BMP-2 could be processed following secretion, hypothesizing that secreted BMP-2 precursors from tumor cells may be processed by cells present in the tumor stroma. Because leukocytes normally infiltrate lung and furin convertase is ubiquitously expressed, the ability of leukocytes to cleave proprotein BMP-2 secreted from A549 cells was examined. First, Applicant determined that the furin convertase is expressed in human leukocytes isolated from whole blood. (Figure 9(c)). Human leukocytes were incubated with A549 cell culture media containing the BMP-2 precursor protein. A Western blot of the incubated media samples was probed with an anti-human BMP-2 precursor antibody that recognizes its C-terminal end. The 45 kD BMP-2 precursor protein was consistently decreased following incubation with the leukocytes (Figure 9(a)). By probing immunoblots with an anti-human BMP-2 antibody that recognizes its N-terminal end, Applicant identified a 31 kD BMP-2 product present only in the media samples incubated with leukocytes. (Figure 9(b)) This data shows that

BMP-2 precursor proteins are cleaved by human leukocytes.

Effect of BMP-2 on Tumors and Cancer Cell Lines

[0097] After determining that BMP-2 was highly expressed in most common cancers, Applicant performed experiments to show that BMP-2 causes cancer invasion
5 and metastasis. Applicant performed experiments with lung cancer cell lines and with nude mice injected with A549 cells.

[0098] The experiments with the nude mice showed that BMP-2 treatment enhances blood vessel formation around tumors from nude mice injected with A549 cells. Some of the mice were co-injected with BMP-2. Gross observations of tissue harvested
10 after six days showed that the addition of recombinant BMP-2 to developing tumors in nude mice caused increased blood vessel formation. (Figure 10) Tissue was also stained with anti-CD 31 antibody which recognizes endothelial cells. A person blind to how the tumors were created then observed the tissue through a microscope and counted the number of vessels that had formed in the tumor. This data showed that BMP-2 caused a
15 statistically significant increase in the number of blood vessels in the tumor. (Figure 11)

[0099] Other studies showed that addition of BMP-2 to cancer cell lines increased expression of vascular endothelial growth factor (VEGF) and the oncogene Sonic Hedgehog. VEGF is the most potent angiogenic factor and is thought to be essential for tumor growth and metastasis. (Folkman, J. J. Nat'l Cancer Inst. 82:4 (1990); Zetter, B.
20 Annual Rev. Med. 49:407 (1998); Ferrara, N. Current Topics Microbiol. Immunol. 237:1 (1999)) Transgenic mice studies have confirmed that overexpression of sonic hedgehog can cause tissue-targeted cancer. (Oro, A.E., et al., "Basal carcinomas in mice overexpressing sonic hedgehog" Science 276: 817-21 (1997)) The addition of

recombinant BMP-2 to human aortic endothelial cells in culture caused an increase in VEGF secretion as determined by ELISA performed on the cell culture media. The concentration of VEGF in the cell culture media before treatment with BMP-2 was 11.2 pg/ml. The VEGF concentration after treatment with 0.500 pg/ml BMP-2 was 233.0 pg/ml and after treatment with 1 ng/ml BMP-2 was 2,969.0 pg/ml. The addition of increasing amounts of BMP-2 to lung A549 lung cancer cells growing in culture also caused a dose responsive increase in the expression of the oncogene Sonic Hedgehog. (Figure 12)

[00100] In addition, Applicants showed that BMP-2 stimulates the migration and invasion of the human lung cancer cell lines A549 and H7249. In one assay, recombinant BMP-2 caused a dose responsive increase in migration of cells from transwell migration chambers. (Figure 13(a)) In another, BMP-2 stimulated the migration of A549 and H7249 cells cultured on glass cover slips toward Affi-blue agarose beads containing recombinant BMP-2. (Figure 13 (c) and (d)) In addition, using transwell chambers coated with Matrigel, Applicants also showed that recombinant BMP-2 caused a dose responsive increase in the invasion of both A549 and H7249 cells. (Figure 13(e))

Effects of Inhibiting BMP-2 Expression

[00101] After finding that BMP-2 enhances cancer invasion and growth, Applicant conducted experiments to determine whether inhibitors of the activity of BMP-2 could be used to treat cancer. In these studies, recombinant mouse noggin (R & D Systems, Minneapolis, MN) was used as a representative inhibitor. Noggin, a natural inhibitor of BMP-2, is a secreted protein that binds BMP-2 and BMP-4, thereby preventing their activation of the BMP receptors. (Weaver, M., et al., Development 126: 4005-4115

(1999); Zimmerman, L.B., et al., Cell 86: 599-606 (1996); Tucker, A.S., et al., Science 282: 1136-1138 (1998); Capdevilla, J., et al., Developmental Biology 197: 205-217 (1998); Brunet, L.J., et al., Science 280: 1455-1447 (1998)) Mouse and human noggin are 98% homologous.

- 5 [00102] The effects of BMP-2 and noggin on tumor growth *in vivo* was examined by co-injecting the A549 cells subcutaneously into nude mice with Affi-Blue agarose beads coated with either albumin, recombinant human BMP-2, or recombinant human noggin. The animals were then sacrificed and tumors measured at 12 or 19 days. Inhibiting BMP-2 activity with noggin resulted in a statistically significant decrease in
- 10 tumor growth. Addition of BMP-2 resulted in a statistically significant increase in tumor growth. (Figure 14)
- [00103] Noggin also decreased the expression of VEGF and sonic hedgehog when added to A549 cells. (Figures 12 and 15)
- [00104] Applicants also found that noggin completely inhibited the ability of
- 15 BMP-2, discussed above, to enhance the migration of the A549 cells in a transwell chamber. (Figure 13(b))

EXAMPLES

Example 1: Identification of BMP-2 Using Representational Difference Analysis

(RDA) Subtraction Technique

- 20 [00105] Representational difference analysis (RDA) subtraction technique was used to identify genes highly expressed in a non-small cell lung carcinoma obtained from a patient (tester) in comparison to normal bronchial human epithelial cells (driver). The technique for RDA described in the following references was followed: Holmes, M.L., et

al., Molecular and Cellular Biology 19: 4182-4190 (1999); Hubank, M., Nucleic Acids Research 22:5640-5648 (1994). Normal human bronchial epithelial cells were purchased from Clonetics, BioWhitaker (Walkersville, Maryland) and were maintained in serum free media. Human tissue specimens were obtained directly from the operating room and immediately frozen in liquid nitrogen. Tissue was stored in liquid nitrogen at -70C.

[00106] To perform RDA, mRNA was purified from the samples using Oligo dT columns (Pharmacia, Peapack, NJ) according to the manufacturer's instructions and cDNA was then obtained using the Pharmacia Time Saver cDNA synthesis kit also according to the manufacturer's instructions. cDNA was digested with Sau3A I endonuclease, R-linker ligated, and amplified by PCR. The R-linkers were removed and J-linkers ligated to the tester. The driver and tester cDNA were hybridized at 67 C for 20 hours (driver in excess 100:1) and the subtracted tester cDNA amplified by PCR. A second round of subtraction was performed using N-linkers (driver in excess 800,000:1). The amplified PCR products were cloned into blue script and sequenced using a IBI Prism 377 DNA sequencer. Known genes corresponding to the subtracted tumor cDNA were identified by a BLAST database search.

Example 2: Detection of Expression of BMP-2 in Human Lung Cancer Specimens Using RT-PCR

[00107] Reverse transcriptase polymerase chain reaction was performed using standard techniques well known in the art. The forward primer was acgagagctctcactgtgcc (SEQ ID No: 15) The reverse primer was cattccggattacatgaggg (SEQ ID No: 16). The chain reaction consisted of denaturation at 95 C for 1 min, annealing at 54 C for 1 min, and extension at 72 C for 2 min with 33 cycles.

**Example 3: Detection of Over-Expression of BMP and BMP Receptors in
Various Cancer Tissue Specimens and Lung Cancer Cell Lines**

[00108] Applicant detected expression of BMP and BMP receptors in a number of normal and cancerous tissue specimens and cells. As described above, all human tissue specimens were obtained directly from the operating room and immediately frozen in liquid nitrogen and stored at -70 C. Normal human bronchial epithelial (NBE) cells were purchased from Clonetics, BioWhitaker (Walkersville, Maryland) and were maintained in serum free media. Immortalized human bronchial epithelial (IHBE), BEAS-2B, cells were derived from normal bronchial epithelial cells immortalized with an adenovirus-12-5V40 hybrid virus (32). A549 and H7249 are highly invasive human lung cancer cell lines. The cell lines were cultured in 5% fetal bovine serum (FBS) in Dulbecco's Modified Eagles medium (DME) containing penicillin, streptomycin, and glutamine with 5% pCO₂ at 37°C. Western blot analysis was used to detect expression of the BMP ligand and its receptors in all of these samples. Immunohistochemistry studies were performed to detect BMP in non-small cell lung carcinoma samples and normal lung tissue samples derived from patients.

Western Blot Analysis

[00109] In preparation for Western blot analysis, cells were lysed in a modified RIPA buffer containing 150 ml NaCl, 50 ml tris, pH 7.5, 1% NP 40, 10% deoxycholic acid, and protease inhibitor cocktail from Calbiochem. Tissue specimens were sonicated on ice in the same modified RIPA buffer. The protein concentration of the resulting samples was measured using the Bradford assay technique. Recombinant human BMP-2, purchased from R & D Systems and reconstituted in PBS with gelatin, served as a

control. Total cellular protein of the samples and recombinant human BMP-2 were analyzed by SDS-PAGE, transferred to nitrocellulose filter (Schleicher and Schuell, Keene, NH) at 35 V for 16 hours at 4°C and then incubated with the desired primary antibody. Specific proteins were detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

[00110] The primary antibodies that were used included mouse anti-human BMP-2, goat anti-human BMP-4, goat anti-human BMP-7, goat anti-human type IA BMP receptor, and goat anti-human type IB BMP-2 receptor. All of these antibodies, except the goat anti-human BMP-7 were purchased from R & D Systems in Minneapolis, MN. The goat anti-human BMP-7 antibody was obtained from Santa Cruz (Santa Cruz, CA).

Immunohistochemistry Analysis

[0100] To perform immunohistochemistry analysis, four micron Cryostat-cut sections were air dried before being fixed in cold acetone for 10 minutes. Sections were washed in cold 0.5 M PBS and intrinsic peroxidase was quenched with 0.03% periodic acid for 20 minutes at room temperature. Sections were then rinsed in cold PBS and 0.5% BSA/PBS was applied to the slides for 15 minutes in a humid chamber. Biotinylated BMP-2/4 (R & D Systems) was applied at a 1:25 dilution in 1% BSA/PBS and incubated overnight at 4°C. Two slides were run as negative controls. One slide was incubated with biotinylated BMP-2 preabsorbed with recombinant human BMP-2 at 1:10 Molar ratio. As a second negative control slide samples were incubated overnight at 4°C with normal rabbit serum. Slides were washed with cold PBS and incubated for one hour in Streptavidin horseradish peroxidase (Dako) at a 1:500 dilution in 1% BSA/PBS. Slides were then counterstained in 0.7% Toluidine Blue.

Example 4: Detection of Processing of Mature BMP-2 by Human

Leukocytes

[0101] Cell culture media from the A549 cells was incubated with leukocytes isolated from whole blood for 16 hours. Then, a Western blot was performed, as
5 described above, on the cell culture media. Mouse anti-human BMP-2 antibody (#MAB355, R & D Systems, Minneapolis, MN) was the primary antibody used to detect the C-terminal end of BMP-2. Goat anti-human BMP-2 (Research Diagnostics, Flanders, NJ) was used to detect the N-terminal end of BMP-2. A Western blot of the leukocytes was also performed with an anti-furin primary antibody to determine that human
10 leukocytes express furin convertase.

Example 5: Analysis of the Effect of BMP-2 and Noggin on Tumor Growth and Tumor Vasculature *In Vivo*

[0102] Nude mice studies were conducted to determine the effect of BMP-2 and one of its inhibitors, noggin, on tumor growth and tumor vasculature. 10^6 A549 cells
15 were injected subcutaneously into nude mice with Affi-Blue agarose beads coated with albumin, recombinant human BMP-2 or recombinant mouse noggin. Both of these recombinant proteins were purchased from R & D Systems and were reconstituted in PBS with gelatin. Coating of Affi-Blue agarose beads with BMP-2 and noggin has been described in the literature. (Abe, E., et al., J. Bone Miner Res. 15: 663-673 (2000);
20 Tucker, A.S., et al., Science 282: 1136-1138 (1998); Zimmerman, L.B., et al., Cell 86: 599-606 (1996)) In brief, 25 ug of Affi-blue agarose beads were incubated with 100 ug/ml albumin, recombinant human BMP-2, or recombinant noggin for 2 hours and then washed 3 times with PBS immediately prior to use. In separate experiments the beads

were not washed prior to injection. The coated beads were injected with the A549 cells into nude mice subcutaneously. To assess tumor growth after 12 or 19 days the length, width, and depth of the tumors were measured in mm. To assess tumor vasculature, tissue including a tumor was harvested after six days. Gross observations of the tissue were made. In addition, the tissue was stained with anti-CD 31 antibody, which recognizes endothelial cells. Vessels in five high power fields were counted by a person blinded to how the tumors were created.

Example 6: Effect of BMP-2 and Noggin on VEGF and Sonic Hedgehog Expression

Western blot analysis of VEGF and sonic hedgehog in presence of BMP-2 and noggin

10 [0103] Western blots, as described above, were performed on total cellular protein samples and cell culture media samples. The primary antibodies used to detect VEGF and sonic hedgehog were anti human VEGF from R & D Systems (Minneapolis, MN) and anti human sonic hedgehog from Santa Cruz (Santa Cruz, CA), respectively.

ELISA of VEGF in presence of BMP-2 and various concentrations of noggin

15 [0104] The sandwich ELISA method was used to determine VEGF concentrations in the cell culture media of A549 cells treated with noggin and in the cell culture media of human aortic endothelial cells treated with BMP-2. 100 ul of the monoclonal capture antibody, diluted in carbonate buffer (sodium bicarbonate, sodium carbonate, pH 9.0), was added to each well of a MaxiSorb Nunc-Immuno plate and
20 incubated overnight at 4 C. The plates were washed two times with washing buffer (1x PBS with 0.0005% tween-20). Then, 200 ul of blocking buffer (1x PBS with 1% BSA) was added per well and incubated for 2 hours at room temperature. The plates were then washed 4 times with washing buffer.

[0105] The recombinant protein standards and samples (100ul per well) were added and the plate was then incubated overnight at 4 C. The plates were washed 5 times with washing buffer. The biotinylated detection antibody was diluted in incubation buffer (1x PBS with 10% fetal bovine serum) for a final concentration of 1 ug/ml. 100 ul of the detection antibody was added per well and incubated for 1 hour on a shaker at room temperature. The plates were washed 6 times with washing buffer and 100 ul of streptavidin-HRP conjugate (1:10,000) was added per well. The plates were incubated for 45 minutes at room temperature on a shaker and then washed 6 times with washing buffer. 100 ul/well of the substrate reagent (0.2 M citrate buffer, 1 mg/ml o-phenylenediamine dihydrochloride (OPG), 3% hydrogen peroxide) was added and covered with aluminum foil for ten minutes. The reaction was stopped with 100 ul/well of 2M sulfuric acid and absorbance determined using an automated plate reader with a 490/690 filter. The protein concentration was then determined from the standard curve.

Example 7: Identification of BMP-2 as a Stimulant of Human Lung Cancer

15 Cell Migration and Invasion

Migration Assay In Monolayer Culture

[0106] To detect BMP-induced migration in a monolayer culture, recombinant human BMP-2 (R & D systems, Minneapolis, MN) was coated to Affi-Blue agarose beads (Bio Rad, Hercules, CA) as described in the literature. (Vainio, S.; et al., Cell 75: 45-58 (1993); Sloan, A.J., et al., Arch Oral Biol. 44: 149-156 (1999)) Briefly, 100 ml of the Affi-Blue agarose beads were incubated with either 10 ml of recombinant BMP2 reconstituted in PBS with gelatin (100 mg/ml) or PBS alone at 37°C for 2 hours, washed with PBS, and reconstituted with 40 ml of PBS. Glass cover slips were coated with

serum free media containing BSA, fibronectin and collagen (32) and 50,000 cells were plated per cover slip in serum free media. Two microliters of the Affi-Blue agarose beads coated with recombinant BMP-2 or dilution buffer were placed in linear fashion next to the cover slips.

5 Chemotactic Assay

[0107] In the chemotactic assay, fifty thousand cells were placed in the upper chamber of an 8 micron transwell migration chamber (Becton Dickinson, Bedford, MA) and 300 ml of serum free media with 0 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant human BMP-2 placed in the lower well. After 24 hours the
10 filters were then removed and the top of the filter wiped with a cotton swab and the cells that migrated through the filters were stained with Syto-16 intercalating dye. Five high power fields were counted using fluorescent microscopy. To show that noggin inhibits BMP-2 induced migration, the experiment was also performed with each of the following in the lower well of the transwell chamber: media alone, recombinant BMP-2 (500
15 ng/ml), and noggin (10 ug/ml) with recombinant BMP-2 (500 ng/ml).

Matrigel Invasion Assay

[0108] Invasion was studied using transwell chambers coated with 100 ml of Matrigel (Becton Dickinson). Fifty thousand cells were placed in the upper chamber and 300 ml of serum free media with 0ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml
20 recombinant BMP-2 placed in the lower wells. After 48 hours the Matrigel was removed and cells that had migrated through the filter were stained with Syto-16 intercalating dye and 5 high power fields counted using fluorescent microscopy.

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2004-16-04-102

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15 Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
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20 Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
165 170 175

25 Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
180 185 190

30 Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
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40 Ile Ser Glu Cys Lys Cys Ser Cys
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96

20 Val Leu Gly Leu Arg Ala Ala Pro Ala Gly Gly Gln His Tyr Leu His
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atc cgc cca gca ccc agc gac aac ctg ccc ttg gtg gac ctc atc gaa
144

25 Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
35 40 45

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192

30 His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
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240

35 Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
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288

40 Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Gly Pro Ala Gly
85 90 95

ggg gcc gag gac ctg gcg gag ctg gac cag ctg ctg cgg cag cgg ccg
336

45 Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
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tcg ggg gcc atg ccg agc gag atc aaa ggg ctg gag ttc tcc gag ggc
384

50 Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
115 120 125

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432

55 Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys

130 135 140

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480

5 Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
145 150 155 160

tgg aat gac cta ggc agc cgc ttt tgg cca cgc tac gtg aag gtg ggc
528

10 Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
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576

15 Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
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624

20 Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
195 200 205

cgg cgc ggg ggt cag cgc tgc ggc tgg att ccc atc cag tac ccc atc
672

25 Arg Arg Gly Gly Gln Arg Cys Gly Trp Ile Pro Ile Gln Tyr Pro Ile
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att tcc gag tgt aag tgt tcc tgc tag
699

30 Ile Ser Glu Cys Lys Cys Ser Cys
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40 <213> Mus musculus

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Ile Arg Pro Ala Pro Ser Asp Asn Leu Pro Leu Val Asp Leu Ile Glu
35 40 45

55

5 His Pro Asp Pro Ile Phe Asp Pro Lys Glu Lys Asp Leu Asn Glu Thr
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10 Leu Leu Arg Ser Leu Leu Gly Gly His Tyr Asp Pro Gly Phe Met Ala
65 70 75 80

15 Thr Ser Pro Pro Glu Asp Arg Pro Gly Gly Gly Gly Gly Pro Ala Gly
85 90 95

20 Gly Ala Glu Asp Leu Ala Glu Leu Asp Gln Leu Leu Arg Gln Arg Pro
100 105 110

25 Ser Gly Ala Met Pro Ser Glu Ile Lys Gly Leu Glu Phe Ser Glu Gly
115 120 125

30 Leu Ala Gln Gly Lys Lys Gln Arg Leu Ser Lys Lys Leu Arg Arg Lys
130 135 140

35 Leu Gln Met Trp Leu Trp Ser Gln Thr Phe Cys Pro Val Leu Tyr Ala
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40 Trp Asn Asp Leu Gly Ser Arg Phe Trp Pro Arg Tyr Val Lys Val Gly
165 170 175

45 Ser Cys Phe Ser Lys Arg Ser Cys Ser Val Pro Glu Gly Met Val Cys
180 185 190

50 Lys Pro Ser Lys Ser Val His Leu Thr Val Leu Arg Trp Arg Cys Gln
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Ile Ser Glu Cys Lys Cys Ser Cys
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<301> Millet, C., Lemaire, P., Orsetti, B., Guglielmi, P., and
Francois, V.

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50 <306> 85-96

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<302> Direct Submission

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ggccccggcc ccctcccgcc gcaccgcccc cgccccggcc ctccgccctc cgcactcccg
180

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cctccctccc tccgcccgt cccgcgccct cctccctccc tcctccccag ctgtcccggt
240

cgcgtc atg ccg agc ctc ccg gcc ccg ccg gcc ccg ctg ctg ctc ctc

45

288

Met Pro Ser Leu Pro Ala Pro Pro Ala Pro Leu Leu Leu Leu
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ggg ctg ctg ctg ctc ggc tcc ccg ccg gcc cgc ggc gcc ggc ccc gag
336

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Gly Leu Leu Leu Leu Gly Ser Arg Pro Ala Arg Gly Ala Gly Pro Glu
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ccc ccc gtg ctg ccc atc cgt tct gag aag gag ccg ctg ccc gtt cgg
384

55

Pro Pro Val Leu Pro Ile Arg Ser Glu Lys Glu Pro Leu Pro Val Arg
35 40 45

5 gga gcg gca ggc tgc acc ttc ggc ggg aag gtc tat gcc ttg gac gag
432
Gly Ala Ala Gly Cys Thr Phe Gly Gly Lys Val Tyr Ala Leu Asp Glu
50 55 60

10 acg tgg cac ccg gac cta ggg gag cca ttc ggg gtg atg cgc tgc gtg
480
Thr Trp His Pro Asp Leu Gly Glu Pro Phe Gly Val Met Arg Cys Val
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528
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80 85 90

20 ggc agg gtc agc tgc aag aac atc aaa cca gag tgc cca acc ccg gcc
576
Gly Arg Val Ser Cys Lys Asn Ile Lys Pro Glu Cys Pro Thr Pro Ala
95 100 105 110

25 tgt ggg cag ccg cgc cag ctg ccg gga cac tgc tgc cag acc tgc ccc
624
Cys Gly Gln Pro Arg Gln Leu Pro Gly His Cys Cys Gln Thr Cys Pro
115 120 125

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672
Gln Glu Arg Ser Ser Ser Glu Arg Gln Pro Ser Gly Leu Ser Phe Glu
130 135 140

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720
Tyr Pro Arg Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro
145 150 155

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768
Gly Ala Glu Glu Arg Ala Arg Gly Asp Gly His Thr Asp Phe Val Ala
160 165 170

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816
Leu Leu Thr Gly Pro Arg Ser Gln Ala Val Ala Arg Ala Arg Val Ser
175 180 185 190

50 ctg ctg cgc tct agc ctc cgc ttc tct atc tcc tac agg cgg ctg gac
864
Leu Leu Arg Ser Ser Leu Arg Phe Ser Ile Ser Tyr Arg Arg Leu Asp
195 200 205

55 cgc cct acc agg atc cgc ttc tca gac tcc aat ggc agt gtc ctg ttt
912

Arg Pro Thr Arg Ile Arg Phe Ser Asp Ser Asn Gly Ser Val Leu Phe
210 215 220

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960
Glu His Pro Ala Ala Pro Thr Gln Asp Gly Leu Val Cys Gly Val Trp
225 230 235

10 cgg gca gtg cct cgg ttg tct ctg cgg ctc ctt agg gca gaa cag ctg
1008
Arg Ala Val Pro Arg Leu Ser Leu Arg Leu Leu Arg Ala Glu Gln Leu
240 245 250

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1056
His Val Ala Leu Val Thr Leu Thr His Pro Ser Gly Glu Val Trp Gly
255 260 265 270

20 cct ctc atc cgg cac cgg gcc ctg gct gca gag acc ttc agt gcc atc
1104
Pro Leu Ile Arg His Arg Ala Leu Ala Ala Glu Thr Phe Ser Ala Ile
275 280 285

25 ctg act cta gaa ggc ccc cca cag cag ggc gta ggg ggc atc acc ctg
1152
Leu Thr Leu Glu Gly Pro Pro Gln Gln Gly Val Gly Gly Ile Thr Leu
290 295 300

30 ctc act ctc agt gac aca gag gac tcc ttg cat ttt ttg ctg ctc ttc
1200
Leu Thr Leu Ser Asp Thr Glu Asp Ser Leu His Phe Leu Leu Leu Phe
305 310 315

35 cga ggg ctg ctg gaa ccc agg agt ggg gga cta acc cag gtt ccc ttg
1248
Arg Gly Leu Leu Glu Pro Arg Ser Gly Gly Leu Thr Gln Val Pro Leu
320 325 330

40 agg ctc cag att cta cac cag ggg cag cta ctg cga gaa ctt cag gcc
1296
Arg Leu Gln Ile Leu His Gln Gly Gln Leu Leu Arg Glu Leu Gln Ala
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1344
Asn Val Ser Ala Gln Glu Pro Gly Phe Ala Glu Val Leu Pro Asn Leu
355 360 365

50 aca gtc cag gag atg gac tgg ctg gtg ctg ggg gag ctg cag atg gcc
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Thr Val Gln Glu Met Asp Trp Leu Val Leu Gly Glu Leu Gln Met Ala
370 375 380

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1440

Leu Glu Trp Ala Gly Arg Pro Gly Leu Arg Ile Ser Gly His Ile Ala
385 390 395

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1488
Ala Arg Lys Ser Cys Asp Val Leu Gln Ser Val Leu Cys Gly Ala Asp
400 405 410

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1536
Ala Leu Ile Pro Val Gln Thr Gly Ala Ala Gly Ser Ala Ser Leu Thr
415 420 425 430

15 ctg cta gga aat ggc tcc ctg atc tat cag gtg caa gtg gta ggg aca
1584
Leu Leu Gly Asn Gly Ser Leu Ile Tyr Gln Val Gln Val Val Gly Thr
435 440 445

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1632
Ser Ser Glu Val Val Ala Met Thr Leu Glu Thr Lys Pro Gln Arg Arg
450 455 460

25 gat cag cgc act gtc ctg tgc cac atg gct gga ctc cag cca gga gga
1680
Asp Gln Arg Thr Val Leu Cys His Met Ala Gly Leu Gln Pro Gly Gly
465 470 475

30 cac acg gcc gtg ggt atc tgc cct ggg ctg ggt gcc cga ggg gct cat
1728
His Thr Ala Val Gly Ile Cys Pro Gly Leu Gly Ala Arg Gly Ala His
480 485 490

35 atg ctg ctg cag aat gag ctc ttc ctg aat gtg ggc acc aag gac ttc
1776
Met Leu Leu Gln Asn Glu Leu Phe Leu Asn Val Gly Thr Lys Asp Phe
495 500 505 510

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1824
Pro Asp Gly Glu Leu Arg Gly His Val Ala Ala Leu Pro Tyr Cys Gly
515 520 525

45 cat agc gcc cgc cat gac acg ctg ccc gtg ccc cta gca gga gcc ctg
1872
His Ser Ala Arg His Asp Thr Leu Pro Val Pro Leu Ala Gly Ala Leu
530 535 540

50 gtg cta ccc cct gtg aag agc caa gca gca ggg cac gcc tgg ctt tcc
1920
Val Leu Pro Pro Val Lys Ser Gln Ala Ala Gly His Ala Trp Leu Ser
545 550 555

55 ttg gat acc cac tgt cac ctg cac tat gaa gtg ctg ctg gct ggg ctt
1968

Leu Asp Thr His Cys His Leu His Tyr Glu Val Leu Leu Ala Gly Leu
560 565 570

5 ggt ggc tca gaa caa ggc act gtc act gcc cac ctc ctt ggg cct cct
2016
Gly Gly Ser Glu Gln Gly Thr Val Thr Ala His Leu Leu Gly Pro Pro
575 580 585 590

10 gga acg cca ggg cct cgg cgg ctg ctg aag gga ttc tat ggc tca gag
2064
Gly Thr Pro Gly Pro Arg Arg Leu Leu Lys Gly Phe Tyr Gly Ser Glu
595 600 605

15 gcc cag ggt gtg gtg aag gac ctg gag ccg gaa ctg ctg cgg cac ctg
2112
Ala Gln Gly Val Val Lys Asp Leu Glu Pro Glu Leu Leu Arg His Leu
610 615 620

20 gca aaa ggc atg gcc tcc ctg ctg atc acc acc aag ggt agc ccc aga
2160
Ala Lys Gly Met Ala Ser Leu Leu Ile Thr Thr Lys Gly Ser Pro Arg
625 630 635

25 ggg gag ctc cga ggg cag gtg cac ata gcc aac caa tgt gag gtt ggc
2208
Gly Glu Leu Arg Gly Gln Val His Ile Ala Asn Gln Cys Glu Val Gly
640 645 650

30 gga ctg cgc ctg gag gcg gcc ggg gcc gag ggg gtg cgg gcg ctg ggg
2256
Gly Leu Arg Leu Glu Ala Ala Gly Ala Glu Gly Val Arg Ala Leu Gly
655 660 665 670

35 gct ccg gat aca gcc tct gct gcg ccg cct gtg gtg cct ggt ctc ccg
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Ala Pro Asp Thr Ala Ser Ala Ala Pro Pro Val Val Pro Gly Leu Pro
675 680 685

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690 695 700

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2400
Asn Thr Cys Phe Phe Glu Gly Gln Gln Arg Pro His Gly Ala Arg Trp
705 710 715

50 gcg ccc aac tac gac ccg ctc tgc tca ctc tgc acc tgc cag aga cga
2448
Ala Pro Asn Tyr Asp Pro Leu Cys Ser Leu Cys Thr Cys Gln Arg Arg
720 725 730

55 acg gtg atc tgt gac ccg gtg gtg tgc cca ccg ccc agc tgc cca cac
2496

Thr Val Ile Cys Asp Pro Val Val Cys Pro Pro Pro Ser Cys Pro His
735 740 745 750

5 ccg gtg cag gct ccc gac cag tgc tgc cct gtt tgc cct gag aaa caa
2544
Pro Val Gln Ala Pro Asp Gln Cys Cys Pro Val Cys Pro Glu Lys Gln
755 760 765

10 gat gtc aga gac ttg cca ggg ctg cca agg agc cgg gac cca gga gag
2592
Asp Val Arg Asp Leu Pro Gly Leu Pro Arg Ser Arg Asp Pro Gly Glu
770 775 780

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2640
Gly Cys Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala Ala Gly Thr Arg
785 790 795

20 tgg cac ccc gtt gtg ccc ccc ttt ggc tta att aag tgt gct gtc tgc
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Trp His Pro Val Val Pro Pro Phe Gly Leu Ile Lys Cys Ala Val Cys
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25 acc tgc aag ggg ggc act gga gag gtg cac tgt gag aag gtg cag tgt
2736
Thr Cys Lys Gly Gly Thr Gly Glu Val His Cys Glu Lys Val Gln Cys
815 820 825 830

30 ccc cgg ctg gcc tgt gcc cag cct gtg cgt gtc aac ccc acc gac tgc
2784
Pro Arg Leu Ala Cys Ala Gln Pro Val Arg Val Asn Pro Thr Asp Cys
835 840 845

35 tgc aaa cag tgt cca gtg ggg tcg ggg gcc cac ccc cag ctg ggg gac
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Cys Lys Gln Cys Pro Val Gly Ser Gly Ala His Pro Gln Leu Gly Asp
850 855 860

40 ccc atg cag gct gat ggg ccc cgg ggc tgc cgt ttt gct ggg cag tgg
2880
Pro Met Gln Ala Asp Gly Pro Arg Gly Cys Arg Phe Ala Gly Gln Trp
865 870 875

45 ttc cca gag agt cag agc tgg cac ccc tca gtg ccc cct ttt gga gag
2928
Phe Pro Glu Ser Gln Ser Trp His Pro Ser Val Pro Pro Phe Gly Glu
880 885 890

50 atg agc tgt atc acc tgc aga tgt ggg gca ggg gtg cct cac tgt gag
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Met Ser Cys Ile Thr Cys Arg Cys Gly Ala Gly Val Pro His Cys Glu
895 900 905 910

55 cgg gat gac tgt tca ctg cca ctg tcc tgt ggc tcg ggg aag gag agt
3024

Arg Asp Asp Cys Ser Leu Pro Leu Ser Cys Gly Ser Gly Lys Glu Ser
915 920 925

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3072
Arg Cys Cys Ser Arg Cys Thr Ala His Arg Arg Pro Ala Pro Glu Thr
930 935 940

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3114
Arg Thr Asp Pro Glu Leu Glu Lys Glu Ala Glu Gly Ser
945 950 955

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35 40 45

10 Ala Gly Cys Thr Phe Gly Gly Lys Val Tyr Ala Leu Asp Glu Thr Trp
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15 His Pro Asp Leu Gly Glu Pro Phe Gly Val Met Arg Cys Val Leu Cys
65 70 75 80

Ala Cys Glu Ala Pro Gln Trp Gly Arg Arg Thr Arg Gly Pro Gly Arg
85 90 95

20 Val Ser Cys Lys Asn Ile Lys Pro Glu Cys Pro Thr Pro Ala Cys Gly
100 105 110

25 Gln Pro Arg Gln Leu Pro Gly His Cys Cys Gln Thr Cys Pro Gln Glu
115 120 125

30 Arg Ser Ser Ser Glu Arg Gln Pro Ser Gly Leu Ser Phe Glu Tyr Pro
130 135 140

35 Arg Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro Gly Ala
145 150 155 160

Glu Glu Arg Ala Arg Gly Asp Gly His Thr Asp Phe Val Ala Leu Leu
165 170 175

40 Thr Gly Pro Arg Ser Gln Ala Val Ala Arg Ala Arg Val Ser Leu Leu
180 185 190

45 Arg Ser Ser Leu Arg Phe Ser Ile Ser Tyr Arg Arg Leu Asp Arg Pro
195 200 205

50 Thr Arg Ile Arg Phe Ser Asp Ser Asn Gly Ser Val Leu Phe Glu His
210 215 220

Pro Ala Ala Pro Thr Gln Asp Gly Leu Val Cys Gly Val Trp Arg Ala
225 230 235 240

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Val Pro Arg Leu Ser Leu Arg Leu Leu Arg Ala Glu Gln Leu His Val
245 250 255

5 Ala Leu Val Thr Leu Thr His Pro Ser Gly Glu Val Trp Gly Pro Leu
260 265 270

10 Ile Arg His Arg Ala Leu Ala Ala Glu Thr Phe Ser Ala Ile Leu Thr
275 280 285

15 Leu Glu Gly Pro Pro Gln Gln Gly Val Gly Gly Ile Thr Leu Leu Thr
290 295 300

20 Leu Ser Asp Thr Glu Asp Ser Leu His Phe Leu Leu Leu Phe Arg Gly
305 310 315 320

Leu Leu Glu Pro Arg Ser Gly Gly Leu Thr Gln Val Pro Leu Arg Leu
325 330 335

25 Gln Ile Leu His Gln Gly Gln Leu Leu Arg Glu Leu Gln Ala Asn Val
340 345 350

30 Ser Ala Gln Glu Pro Gly Phe Ala Glu Val Leu Pro Asn Leu Thr Val
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35 Gln Glu Met Asp Trp Leu Val Leu Gly Glu Leu Gln Met Ala Leu Glu
370 375 380

Trp Ala Gly Arg Pro Gly Leu Arg Ile Ser Gly His Ile Ala Ala Arg
385 390 395 400

40 Lys Ser Cys Asp Val Leu Gln Ser Val Leu Cys Gly Ala Asp Ala Leu
405 410 415

45 Ile Pro Val Gln Thr Gly Ala Ala Gly Ser Ala Ser Leu Thr Leu Leu
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435 440 445

55 Glu Val Val Ala Met Thr Leu Glu Thr Lys Pro Gln Arg Arg Asp Gln
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Arg Thr Val Leu Cys His Met Ala Gly Leu Gln Pro Gly Gly His Thr
465 470 475 480

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485 490 495

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515 520 525

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Pro Pro Val Lys Ser Gln Ala Ala Gly His Ala Trp Leu Ser Leu Asp
545 550 555 560

25 Thr His Cys His Leu His Tyr Glu Val Leu Leu Ala Gly Leu Gly Gly
565 570 575

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580 585 590

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595 600 605

40 Gly Val Val Lys Asp Leu Glu Pro Glu Leu Leu Arg His Leu Ala Lys
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675 680 685

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690 695 700

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725 730 735

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740 745 750

25 Gln Ala Pro Asp Gln Cys Cys Pro Val Cys Pro Glu Lys Gln Asp Val
755 760 765

30 Arg Asp Leu Pro Gly Leu Pro Arg Ser Arg Asp Pro Gly Glu Gly Cys
770 775 780

35 Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala Ala Gly Thr Arg Trp His
785 790 795 800

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805 810 815

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820 825 830

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835 840 845

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850 855 860

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900

905

910

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OC-99654.1

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5 <302> DRM/Gremlin (CKTSF1B1) maps to human chromosome 15 and is highly expressed in adult and fetal brain

<303> Cytogenet. Cell Genet.

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<301> Topol, L.Z., Marx, M., Calothy, G., and Blair, D.G.

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<301> Lah, M., Brodnicki, T., Maccarone, P., Nash, A., Stanley, E.,
and Harvey, R.P.

5 <302> Human cerberus related gene CER1 maps to chromosome 9

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672
Cys Leu Pro Ala Lys Phe Thr Thr Met His Leu Pro Leu Asn Cys Thr
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 <301> ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., and Heldin, C.H.
 25
 <302> Characterization of type I receptors for transforming growth factor-beta and activin
 30 <303> Science
 <304> 264
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 35 <306> 101-104
 <307> 1994
 40 <308> NM_001203
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 50 <301> Ide, H., Katoh, M., Sasaki, H., Yoshida, T., Aoki, K., Nawa, Y., Osada, Y., Sugimura, T., and Terada, M.
 <302> Cloning of human bone morphogenetic protein type IB receptor (BMPR-IB) and its expression in prostate cancer in comparison with
 55 other BMPRs

- 5 <303> Oncogene
- <304> 14
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- <306> 1377-1382
- 10 <307> 1997
- <308> NM_001203
- <309> 2000-10-31
- 15 <313> (1)..(2032)
- 20 <300>
- <301> Ide, H., Saito-Ohara, P., Ohnami, S., Osada, Y., Ikeuchi, T.,
Yoshida, T., and Terada, M.
- 25 <302> Assignment of the BMPR1A and BMPR1B genes to human chromosome
10q22.3 and 4q23-->q24 by in situ hybridization and radiation hybrid
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- 30 <303> Cytogenet. Cell. Genet.
- <304> 81
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- <307> 1998
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- 40 <309> 2000-10-31
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- <301> Astrom, A.K., Jin, D., Imamura, T., Roijer, E., Rosenzweig, B.,
Miyazono, K., ten Dijke, P., and Stenman, G.
- 50 <302> Chromosomal localization of three human genes encoding bone
morphogenetic protein receptors
- 55 <303> Mamm. Genome

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 1902
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<222> (367)..(606)

<223> Activin_recip; Region: Activin types I and II

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<222> (883)..(1746)

10 <223> pkinase; Region: Eukaryotic protein kinase domain

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<222> (883)..(1746)

<223> TyrKc; Region: Tyrosina kinase, catalytic domain

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25 <222> (883)..(1725)

<223> TKc; Region: Serine/Threonine protein kinases, catalytic domain

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35 40 45

Thr Asp Gly Tyr Cys Phe Thr Met Ile Glu Glu Asp Asp Ser Gly Leu
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45 Pro Val Val Thr Ser Gly Cys Leu Gly Leu Glu Gly Ser Asp Phe Gln
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50 Cys Arg Asp Thr Pro Ile Pro His Gln Arg Arg Ser Ile Glu Cys Cys
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55 Thr Glu Arg Asn Glu Cys Asn Lys Asp Leu His Pro Thr Leu Pro Pro

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15	Phe Cys Tyr Phe Arg Tyr Lys Arg Gln Glu Thr Arg Pro Arg Tyr Ser		
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20	Ile Gly Leu Glu Gln Asp Glu Thr Tyr Ile Pro Pro Gly Glu Ser Leu		
	165	170	175
25	Arg Asp Leu Ile Glu Gln Ser Gln Ser Ser Gly Ser Gly Ser Gly Leu		
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30	Pro Leu Leu Val Gln Arg Thr Ile Ala Lys Gln Ile Gln Met Val Lys		
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35	Gln Ile Gly Lys Gly Arg Tyr Gly Glu Val Trp Met Gly Lys Trp Arg		
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40	Gly Glu Lys Val Ala Val Lys Val Phe Phe Thr Thr Glu Glu Ala Ser		
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45	Trp Phe Arg Glu Thr Glu Ile Tyr Gln Thr Val Leu Met Arg His Glu		
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50	Asn Ile Leu Gly Phe Ile Ala Ala Asp Ile Lys Gly Thr Gly Ser Trp		
	260	265	270
55	Thr Gln Leu Tyr Leu Ile Thr Asp Tyr His Glu Asn Gly Ser Leu Tyr		
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60	Asp Tyr Leu Lys Ser Thr Thr Leu Asp Ala Lys Ser Met Leu Lys Leu		
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65	Ala Tyr Ser Ser Val Ser Gly Leu Cys His Leu His Thr Glu Ile Phe		
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Ser Thr Gln Gly Lys Pro Ala Ile Ala His Arg Asp Leu Lys Ser Lys
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10 Leu Ala Val Lys Phe Ile Ser Asp Thr Asn Glu Val Asp Ile Pro Pro
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25 Gly Gly Ile Val Glu Glu Tyr Gln Leu Pro Tyr His Asp Leu Val Pro
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2004-12-01 10:00